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# FREE D-AMINO ACIDS IN THE TISSUES OF MARINE BIVALVES

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## Abstract

Seventeen species of marine bivalves were surveyed for the presence of free D-alanine, D-aspartate, and D-valine in their tissues. D-aspartate was found in several species in concentrations approaching those of L-aspartate. D-alanine was detected—particularly in lucinid and vesicomyid clams—at levels exceeding manyfold those of L-alanine. D-valine was absent in all cases. A test of a hydrolysate of bulk soluble proteins of *Lucinoma aequizonata*, a species characterized by extremely high levels of D-alanine, showed no major incorporation of D-alanine into proteins. The implications of these results, for previously published analytical data and for human nutrition, are discussed.

## Introduction

D-amino acids generally are viewed as natural oddities. They are usually not found in proteins, and they occur only occasionally in sizable quantities, either freely dissolved, or incorporated into peptides and metabolites in the tissues of animals and plants (Robinson, 1976; Bodanszky and Perlman, 1969; Corrigan, 1969). However, it has been suggested that the D-isomers of amino acids were as common as the L-forms in ancient prebiotic times on earth. Since the L-isomers were used exclusively by various life forms, they were removed from this natural equilibrium. The supply of L-amino acids was maintained by chemical racemization from the D-isomers left behind (Aono and Yuasa, 1977). Even today relatively large amounts of D-amino acids can be identified in oceanic waters, where apparently they have been formed by chemical racemization from the large pool of dissolved L-amino acids (Lee and Bada, 1977).

Widespread attention was first focused on D-amino acids when they were proposed to be causes and indicators of cancer. Proteins in tumor tissues were thought to contain high levels of D-glutamic acid, thus distinguishing them from normal tissue (Koegl and Erxleben, 1939). This theory was rejected, however, after years of controversy (Miller, 1950).

Recently, the occurrence of D-aspartate instead of the L-form in some proteins has been a focus of investigation for molecular repair mechanisms (McFadden and Clarke, 1982). It was reported that methylated aspartyl residues in erythrocyte membrane proteins had been converted to the D-form. According to the theory, the appearance of D-aspartate in proteins is a first sign of degradation. These proteins are either tagged for disposal, or the D-aspartyl residue can be reversed to the L-form after methylation.

A similar mechanism, time-dependent chemical racemization of aspartate within

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proteins with a low turnover rate, like eye lens or dental proteins, has been used to date these proteins by measuring the ratio of the D- to the L-form of aspartate (Masters, 1983; Bada and Brown, 1980). Exactly the same principle of racemization has also been used extensively to determine, with great accuracy, the ages of fossils, since the normally present L-aspartate racemizes chemically at a constant rate after the death of an organism (Bada and Schroeder, 1975).

D-aspartate may also be a neurotransmitter (Wiklund *et al.*, 1982). Presently, it is being used experimentally as a non-metabolizable replacement for L-glutamate and L-aspartate in neurotransmitter research, since it can use the same uptake sites (Drejer *et al.*, 1983; Taxt and Storm-Mathisen, 1984).

Since finding that the artificial sweetener aspartame produces D-aspartate when heated (*e.g.*, during cooking) another line of research has been initiated (Boehm and Bada, 1984). Thus humans may be exposed nutritionally to considerable amounts of a D-amino acid due to increased consumption of aspartame worldwide.

Only a few publications have focused on the metabolic role of free D-amino acids in animal and plant tissue. D-alanine is present in some molluscs (Matsushima *et al.*, 1984) and crustaceans (D'Aniello and Giuditta, 1980), and it has been demonstrated to be synthesized during anaerobic metabolism in annelids (Felbeck, 1980; Schoettler *et al.*, 1983). Recently, a study of D-amino acids, as indicated by the reaction with D-amino acid oxidase, in a variety of marine invertebrates was published (Preston, 1987a). D-amino acids were found in 18 of the 43 species of the 8 phyla surveyed. The presence and metabolism of D-aspartate has been investigated in the tissues of the bivalve *Solemya reidi* (Felbeck, 1985) and of some cephalopods (D'Aniello and Giuditta, 1977, 1978). In all cases, the D- and the L-forms were present in about equal concentrations. The concentrations of D- and L-alanine in the polychaete *Arenicola marina* are approximately the same, and the concentrations of the two isomers increase similarly in response to metabolic stress (Felbeck, 1980; Schoettler *et al.*, 1983). The bivalve *Solemya reidi* takes up D-aspartate from environmental seawater and metabolizes it just as quickly as it does the L-form. Initially the D-form is converted into the L-isomer before further metabolism takes place (Felbeck, 1985). The uptake and metabolism of D-alanine from seawater has been described recently for coelomocytes of the annelid *Glycera dibranchiata* (Preston, 1987b).

Marine invertebrates commonly have extremely high concentrations of free amino acids which, in the event of osmotic stress, form the largest share of the pool of intracellular osmolytes (Bishop *et al.*, 1983). Therefore, D-amino acids in this pool might serve as important metabolic reserves, sinks, or regulatory factors.

Several investigators have described either isolated occurrences of individual D-amino acids in some invertebrates or have measured the unspecified presence of D-amino acids. No known publication has surveyed organisms for individual D-amino acids. The recent availability of chromatographic screening techniques for some amino acid isomers prompted our investigation of a number of marine bivalves for the presence of specific D-amino acids. We chose to study the Bivalvia because their physiology is well known, a variety of species is readily available, and their tissues contain high concentrations of free amino acids.

## Materials and Methods

### *Animals*

Animals were purchased live at fish markets or collected from a variety of locations (Table I). The animals from the Santa Barbara channel were collected shipboard

TABLE I

*Collection areas of animals*

| Animal | Collection area |
|---|---|
| *Bathymodiolus thermophilus* | Pacific, Galapagos hydrothermal vents |
| *Calyptogena elongata* | Pacific, Santa Barbara channel |
| *Chione californiensis* | Pacific, Gulf of California |
| *Chione stutchburyi* | Fish market, San Diego, California |
| *Codakia obicularis* | Atlantic, Bahamas, intertidal |
| *Codakia tigerina* | Fish market, Phillipines |
| *Corbicula fluminea* | Fish market, San Diego |
| *Crassostrea virginica* | Fish market, San Diego |
| *Hiatella pholadis* | Pacific, La Jolla, California |
| *Hinnites multirugosus* | Pacific, San Diego |
| *Lima hemphilli* | Pacific, San Diego |
| *Lucinoma aequizonata* | Pacific, Santa Barbara channel |
| *Mercenaria mercenaria* | Fish market, San Diego |
| *Modiolus capax* | Pacific, San Diego |
| *Mytilus edulis* | Fish market, San Diego |
| *Solemya reidi* | Pacific, Santa Monica Bay |
| *Tapes japonica* | Fish market, San Diego |

by otter-trawl. *Solemya reidi* was collected shipboard in Santa Monica Bay by Van Veen grab. Most other species were collected by divers and were maintained alive in flow-through seawater tanks, at approximate *in situ* temperatures, for a maximum of ten days before being sacrificed. *Bathymodiolus thermophilus* specimens were collected by the submarine DSRV "Alvin" during a cruise to the Galapagos hydrothermal vents. The animals were frozen upon retrieval. *Codakia tigerina* was purchased alive at a fish market in the Philippines and then shipped by air freight in 70% alcohol.

*Sample preparation*

To account for the presence of symbiotic bacteria in the gills of some of the bivalves used in this study (Felbeck *et al.*, 1981; Felbeck, 1983), all bivalves were opened, and the gills were removed and analyzed separately from the remaining soft parts.

The tissue, frozen with liquid nitrogen, was first pulverized in a mortar. The homogenization was then completed in 1 $N$ $HClO_4$ with an Ultra-Turrax homogenizer. The homogenate was centrifuged at 12,000 $\times$ $g$ for 15 min, and the supernatant was neutralized with 3 $M$ $KHCO_3$. The resulting precipitate was removed by centrifugation. An aliquot of this extract was derivatized with o-phthaldialdehyde (OPA) and N-acetyl-L-cystein (NAC), according to the method described by Aswad (1984). The amino acid isomers were then separated on a C18 reverse phase column with a gradient of 50 m$M$ sodium acetate, pH 5.8, containing 8% methanol (Sol. A) to methanol (Sol. B). The gradient was (in % of solution B): 0 min, 0%; 4 min, 0%; 10 min 25%; 20 min 27%; 34 min, 52%; and 50 min, 52%. Using this gradient—which was modified from the one described by Aswad (whose sole purpose was to separate D-and L-aspartate)—the two alanine and valine isomers could also be separated completely.

Using a Gilson Datamaster integrator, standards for the D- and L-isomers of aspartate, valine, and alanine were used to determine standard response curves. When samples were analyzed, the area under each individual peak was used to determine concentration and, subsequently, the ratio of the individual stereoisomers.

To determine whether D-alanine was present in the proteins of *L. aequizonata,* tissue of a whole animal was homogenized in distilled water with an Ultra Turrax. After centrifugation, the pellet was twice resuspended and rehomogenized in water. The combined supernatants were dialyzed against multiple changes of distilled water for five days to remove all free amino acids. The resulting solution of mixed soluble proteins of *L. aequizonata* was then precipitated with perchloric acid, centrifuged, and the pellet hydrolyzed overnight with HCl. The hydrolyzate was then analyzed for D-amino acids as described above.

## Results

Significant concentrations of D-aspartate and D-alanine and their L-isomers were detected (Table II); no D-valine was found. All Lucinidae showed high concentrations of D-alanine—concentrations much higher than those of L-alanine. D-alanine also was detected in *Mercenaria mercenaria,* both species of *Chione, Hinnites giganteus, Lima hemphilli* (gills), *Bathymodiolus thermophilus, Crassostrea virginica* (gills), *Tapes japonica, Hiatella pholadis,* and *Corbicula fluminea.* In all of these species, the concentration ratios of D- to L-isomer was below one. In the Mytilidae *Mytilus edluis* and *Modiolus capax,* no D-alanine was detected, but D-aspartate was found in concentrations approaching those of the L-isomer. D-aspartate was also detected in the gills of *Bathymodiolus thermophilus.*

No D-alanine was detected in the hydrolyzed soluble protein fraction of *Lucinoma aequizonata.*

Because amino acid levels among individual animals of the same species are typically highly variable, we did not attempt to establish average concentrations for a large number of bivalves but instead focused on the presence of D-amino acids. We postulate that the detection of D-amino acids in any individual organism is significant for the species in general.

## Discussion

The lucinids contained the highest D- to L-ratio of alanine. The extremely high level of free alanine in *Codakia obicularis* tissues has been measured only by ion-exchange amino acid analysis and, therefore, has been attributed entirely to "generic" alanine acting as an osmoregulatory agent or an end-product of anaerobic metabolism (Berg and Alatalo, 1984). In fact, most of this alanine is in the D-form, prompting us to question the function of the D-alanine in this bivalve as well as in all lucinid clams. One possibility is that the D-alanine is entirely made by the symbiotic bacteria inside the cells of the gill. Gram negative bacteria, like the symbiotic species found in the gill (see Schweimanns and Felbeck, 1985, for review), often contain D-alanine in their cell walls (Katz and Derrain, 1977). Therefore, extraction of the cell wall could yield significant amounts of the D-isomer of alanine. In addition, these bacteria are thought to provide a major share of the bivalves' nutritional needs by fixing $CO_2$ from the seawater and transferring reduced organic compounds, possibly including D-alanine, to the host. It is unlikely that the D-amino acids originate in bacteria, however, since tissues lacking bacteria have a D- to L-isomer ratio similar to that of gills densely populated with bacteria. If the bacteria produce and export D-alanine, then the gill preparations should show a larger share of the D-isomer. In addition, some bivalve species (Table II) without symbiotic bacteria also have high concentrations of D-alanine.

Another peculiar aspect of the large proportion of D-alanine in the free amino

TABLE II

*Concentrations of D- and L-amino acids in the tissues of marine bivalves*

| Animal | Tissue | n | L-alanine (umol/g fw) (x ± SD) | D-alanine (umol/g fw) (x ± SD) | Ratio D/L | L-aspartate (umol/g fw) (x ± SD) | D-aspartate (umol/g fw) (x ± SD) | Ratio D/L |
|---|---|---|---|---|---|---|---|---|
| SOLEMYIDAE | | | | | | | | |
| *Solemya reidi* | foot | 2 | 14.2 ± 6.9 | 0.1 ± 0.1 | 0.01 | 13.3 ± 2.4 | 12.4 ± 1.6 | 0.93 |
| | gill | 2 | 3.9 ± 0.7 | 1.4 ± 0.6 | 0.36 | 7.6 ± 2.0 | 5.4 ± 2.2 | 0.71 |
| MYTILIDAE | | | | | | | | |
| *Mytilus edulis* | foot | 2 | 4.4 ± 0.2 | — | — | 4.6 ± 1.8 | 1.4 ± 0.5 | 0.3 |
| | gill | 2 | 2.8 ± 0.9 | — | — | 3.9 ± 0.8 | 3.5 ± 0.7 | 0.9 |
| *Modiolus capax* | foot | 2 | 2.7 ± 1.4 | 4 ± 5.7 | 1.5 | 6.9 ± 2.7 | 2.6 ± 0.01 | 0.38 |
| | gill | 1 | 0.5 | 0.7 | 1.4 | 2.9 | 2.3 | 0.79 |
| *Bathymodiolus thermophilus* | foot | 1 | 12.3 | 0.9 | 0.08 | 2.4 | — | — |
| | gill | 1 | 11.4 | 3.2 | 0.28 | 2.2 | 0.7 | 0.31 |
| OSTREIDAE | | | | | | | | |
| *Crassostrea virginica* | mantle | 1 | 19.4 | — | — | 3.5 | — | — |
| | gill | 2 | 9.9 ± 2.6 | 1.1 ± 0.1 | 0.12 | 5.3 ± 0.5 | — | — |
| PECTINIDAE | | | | | | | | |
| *Hinnites multirugosus* | foot | 2 | 1.4 ± 1.2 | 1.1 ± 0.9 | 0.79 | 1.0 ± 1.3 | 0.4 ± 0.6 | 0.4 |
| | gill | 2 | 0.5 ± 0.2 | 0.7 ± 0.9 | 1.4 | 0.5 ± 0.2 | 0.2 ± 0.2 | 0.4 |
| LIMIDAE | | | | | | | | |
| *Lima hemphilli* | gill | 2 | 1.0 ± 0.5 | 0.5 ± 0.3 | 0.5 | 0.8 ± 0.1 | — | — |
| VENERIDAE | | | | | | | | |
| *Tapes japonica* | foot | 1 | 13.4 | 11.2 | 0.84 | 15.5 | — | — |
| | gill | 1 | 4.7 | 3.5 | 0.74 | 1.7 | 0.2 | 0.12 |
| *Chione californiensis* | foot | 1 | 3.0 | 2.1 | 0.7 | 14.6 | — | — |
| | gill | 3 | 3.8 ± 2.6 | 1.8 ± 1.5 | 0.47 | 4.5 ± 0.9 | — | — |
| *Chione stutchburyi* | foot | 1 | 4.6 | 2.0 | 0.44 | 6.6 | — | — |
| | gill | 2 | 3.3 ± 1.5 | 1.3 ± 0.7 | 0.39 | 3.3 ± 2.8 | — | — |
| *Mercenaria mercenaria* | foot | 2 | 17.7 ± 3.1 | 16.2 ± 1.9 | 0.92 | 7.8 ± 3.5 | — | — |
| | gill | 3 | 4.2 ± 2.2 | 2.8 ± 1.4 | 0.67 | 4.1 ± 1.4 | — | — |
| CORBICULIDAE | | | | | | | | |
| *Corbicula fluminea* | foot | 2 | 1.9 ± 0.4 | 0.9 ± 1.2 | 0.47 | 0.7 ± 0.2 | — | — |
| | gill | 3 | 3.8 ± 0.8 | 0.6 ± 0.1 | 0.16 | 0.4 ± 0.3 | — | — |
| HIATELLIDAE | | | | | | | | |
| *Hiatella pholadis* | gill | 2 | 4.4 ± 1.0 | 1.4 ± 0.2 | 0.32 | 2.3 ± .01 | — | — |
| VESICOMYIDAE | | | | | | | | |
| *Calyptogena elongata* | foot | 1 | 1.1 | 11.3 | 10.45 | 11.5 | — | — |
| | gill | 1 | 0.3 | 2.7 | 9.0 | 1.4 | — | — |
| LUCINIDAE | | | | | | | | |
| *Codakia obicularis* | foot | 1 | 13.5 | 187.2 | 13.9 | 0.3 | 0.2 | 0.67 |
| | gill | 1 | 2.4 | 26.6 | 11.1 | 0.3 | 0.1 | 0.33 |
| *Codakia tigerina* | foot | 1 | 0.4 | 22.4 | 56.0 | 0.6 | 0.1 | 0.17 |
| | gill | 1 | 0.4 | 21.5 | 53.7 | 0.4 | — | — |
| *Lucinoma aequizonata* | foot | 1 | 3.8 | 84.2 | 22.2 | 0.4 | 0.3 | 0.8 |
| | gill | 3 | 3.1 ± 0.5 | 26.1 ± 4.1 | 8.4 | 1.0 ± 0.9 | 0.1 | 0.1 |

The levels are given in μmol/gram fresh weight with the standard deviation. When only gills were tested, the foot was too small to be easily dissected and analysed. ("—" below 0.1 μmol/g fw)

acids of Lucinidae is that the enzyme most commonly responsible for the formation of D-amino acids—amino-acid racemase—would cause an equal distribution between the two isomers (Barman, 1969). The fact that up to 98% of the free alanine pool is in the D-form suggests that: (a) another specialized enzyme is responsible for the metabolism of the D-isomer; and (b) the D-isomer is not metabolized after conversion to the L-form by a racemase, but used separately. Aside from the lucinid clams, however, other examples were found where the ratio of D- to L-alanine was lower: below one. Here we assume that a racemase interconverts the two isomers.

The occurrence of D-aspartate can be explained more easily by the presence of a

racemase, for the maximal ratio of the D- to the L-isomer was around one. Indeed, this specific racemase has already been demonstrated in *Solemya reidi* (Felbeck, 1985). The aspartate-racemase does not catalyze the conversion of alanine.

In spite of the high D-alanine concentration in *Lucinoma aequizonata,* no detectable quantities of D-alanine were found in the proteins of this animal. Therefore, the selection for the L-isomer of alanine in protein synthesis is significant. Since the method used only provides a crude overview of a selected group of proteins—those soluble in distilled water—we cannot exclude the possibility that some minor fraction of the soluble or insoluble proteins would include D-alanine; neither of these would have been detected by the method used.

Wide ranging surveys for the presence of D-amino acids are rarely in the literature. The review article by Corrigan (1969) and Preston's (1987a) recent results are the only known examples. Certainly, one reason is that simple, quick methods to determine the concentrations of D- and L-isomers of individual amino acids have been published only recently. Before this, either both isomers were detected as a sum (*e.g.,* in HPLC with OPA/mercaptoethanol derivatization or with the classical ion exchange amino acid analyzer) or just the L-isomer was detected in typically stereospecific enzymatic determinations. Since it was always assumed that no D-amino acids were present, the results obtained by these methods were taken as representative for "all" amino acids. The D-amino acids concentrations found by Preston (1987a) were obtained unspecifically with a test using D-amino acid oxidase and, therefore, were only applicable as indicator of the general presence of most D-amino acids (D-aspartate and D-glutamate do not react with the D-amino acid oxidase).

Our survey includes the amino acids alanine and aspartate, both of which are commonly found in high concentrations in marine invertebrates, and shows the frequent occurrence of both stereoisomers.

This result is significant for "standard" experimental research organisms like *Mytilus edulis* (Bishop *et al.,* 1983). In this species, the pool of free aspartate is used as an initial substrate for anaerobic metabolism (see de Zwaan and Putzer, 1985, for review). Whenever the concentration of this amino acid was tested using enzymatic methods, only about half of the available amino acid was detected; *i.e.,* the pool of aspartate was actually higher than measured. This may explain the apparent lack of enough initial substraate for anaerobic energy metabolism, as recently reviewed by de Zwaan and Putzer (1985). Similarly, in other organisms such as the polychaete *Arenicola marina,* the initial depletion of the (enzymatically measured) L-aspartate is not large enough (Felbeck, 1980; Schoettler *et al.,* 1983). We think it is possible that D-aspartate, as well as the L-isomer, occurs in *Arenicola,* and that it serves there as additional substrate not detected by enzymatic analysis which after rapid racemization also can be used as metabolic substrate.

We conclude that many published results where amino acid levels in invertebrates have been used as indicators for metabolic pathways, or to calculate metabolic rates, will have to be reassessed because D-amino acids may be present in the tissues used.

Currently, we can only speculate what the metabolic role of D-amino acids is in marine invertebrates. Amino acids are usually used as osmolytes in the tissues of marine invertebrates and, therefore, are often present in very high concentrations (Bishop, 1983; Yancey *et al.,* 1983). The exchange of part or most of the L-isomer for the D-isomer may influence regulatory mechanisms involving these amino acids. Glutamate-pyruvate-transaminase is inhibited by high levels of L-alanine (Barman, 1969); the D-isomer may not have this effect on this enzyme.

Finally, large quantities of free D-amino acids in tissues of common marine bi-

valves may affect human health. Some of our test species were obtained from commercial fish markets. For example, large quantities of D-alanine were found in *Codakia tigerina* specimens bought in a fish market in the Philippines. These fish are routinely consumed by humans. Even *Mytilus edulis*, one of the most common bivalves cultured and consumed in large quantities, contains a concentration of D-aspartate, equal to that of L-aspartate, which is usually between 3 and 14 μmol/g fresh weight (de Zwaan and Putzer, 1985). Little research has been done on the metabolism of D-amino acids in humans or on the effect of long-term exposure to D-amino acids. D-amino acids can cause analgesia in humans, and some D-amino acids are powerful inhibitors of some enzymes involved in regular metabolic pathways (Koyuncuoglu and Berkman, 1982). The result presented in this paper—that some D-amino acids exist sometimes in extremely high concentrations in commonly consumed shellfish—should prompt a closer examination of the effects of D-amino acids on humans.

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